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Chapter 1: Introduction

1.1. Cancer and its epidemiology

Development of cancer is a multi-step process and results in abnormal cells characterized by uncontrolled proliferation with the capacity to invade and destroy normal body tissue. Hanahan and Weinberg have defined the hallmarks of cancer which describes the different capabilities of tumor cells such as evasion of growth suppressors, inducing angiogenesis, genome instability and mutation, enabling replicative immortality, so on and so forth [1,2]. Tumorigenesis i.e., the expression of malignant phenotype in normal cells is a complex process and involves several steps such as initiation, promotion, malignant conversion and tumor progression [3]. Clinical staging of cancer involves the use of physical exams, imaging scans, diagnostic tests, and biopsy to determine the stage of cancer using different methods such as TNM staging, cancer stage grouping etc. According to Globocan 2020, 19.3 million new cases were reported worldwide from which Asia accounts for 49.3% incidence and India accounts for 1.3 million cases [4].

1.2. Treatment of cancer:

Traditional forms of cancer treatment include:

- Surgery: involves physical removal of a tumor
- Radiation therapy: makes use of ionizing radiation in high doses to shrink and kill cancer cells
- Chemotherapy: is the use of drugs to kill cancer cells

More recently developed treatments include immunotherapy, precision therapy, hormone therapy and targeted therapy [5]. However, there are several side-effects associated with traditional methods whereas the development of resistance and heterogeneity of cancer limits other forms of treatment [6].

1.3. Introduction to Nanotechnology:

Nanotechnology is the study of materials having at least one dimension in the 1-100 nm range. Behavior of materials in this size range varies greatly with respect to the electrical, morphological, magnetic and chemical properties as compared to their bulk counterparts, thus changing the interplay between them and biomolecules. An increase in the surface area:volume ratio is also observed which leads to higher surface reactivity. Apart from these properties, synthesis of nanoparticles can be performed using a variety of materials including organic and inorganic, in various sizes and shapes. High surface reactivity can be exploited for surface

functionalization using targeting moieties which are over-expressed in diseases or coating with different polymers which can confer more favorable properties like stimulus responsiveness. Variable tuning of size and shape can also be performed as per required application [7,8,9]. As a result of these properties, limitations of conventional therapies such as non-selectivity, low solubility, toxicity, poor biodistribution and low therapeutic index can be overcome using nanoparticles [10]. Thus, nanotechnology has the potential to ameliorate cancer disease management by improving diagnostic and treatment modalities. For diagnostic applications nanoparticles have been used as contrast agents in techniques like magnetic resonance imaging, computed tomography, positron emission tomography and also light emitting agents as is the case in optical imaging techniques [11]. In order to impart therapy, nanoparticles have been used for the delivery of micro- and macro- therapeutic molecules and also as mediators of photodynamic therapy, photothermal therapy, sonodynamic therapy, radiation therapy etc [12]. Advancements in nanotechnology has also led to the development of nanoparticles capable of diagnosis and therapy simultaneously giving rise to theranostic nanoparticles.

1.1 Nanoparticles in cancer therapy

Nanoparticles have been used in multiple ways to improve the clinical outcome of cancer therapy. Some examples are listed below:

- Limitations of traditional chemotherapy can be overcome by the development of targeted and stimulus responsive drug delivery systems. These properties have been extended for delivery of immunostimulatory agents, chemokines and cytokines thus benefitting immunotherapy [13]
- Nanoparticles used as radiosensitizers have shown longer circulation time and amplification of radiation dose locally [14]
- Ability of nanoparticles to generate heat or reactive oxygen species has translated into photothermal and photodynamic therapies [15]

Chapter 2: Review of Literature

2.1. Types of nanoparticles:

The different types of nanoparticles studied for cancer therapy can be broadly classified into organic, inorganic and a new emerging class of biomimetic nanoparticles. Organic nanoparticles are prepared using polymers and lipids and include solid lipid nanoparticles, liposomes, micelles and polymeric nanoparticles. Organic nanoparticles possess very good

biocompatibility as a result of which they constitute a majority of clinically approved cancer nanomedicine and are majorly used for delivery applications [16]. Inorganic nanoparticles consist of an expansive range of nanomaterials synthesized using elemental metals, metal salts and oxides such as gold, iron oxide, copper sulfide nanoparticles, quantum dots, mesoporous silica nanoparticles etc. Approaching the nanoscale, inorganic nanoparticles demonstrate novel physical properties (discussed in section 2.3) leading to their implementation in therapeutic and imaging applications [17]. Biomimetic nanoparticles combine synthetic material flexibility with the features of biological materials and examples include cell membrane coated nanoparticles, viral capsid protein derived nanoparticles etc. Self-assembled DNA origami molecules with distinct and homogenous geometries are also being developed [18].

2.2. Organic and inorganic nanoparticles in cancer treatment:

Several groups have used organic nanoparticles for delivery of cargo such as chemotherapeutic drugs, imaging agents, photothermal agents etc. Organic nanoparticles extensively employed include liposomes, dendrimers, solid lipid nanoparticles, polymeric micelles and polymerosomes. Doxil was the first FDA approved cancer nanomedicine which consisted of PEGylated liposomes to prevent immune clearance and used for the delivery of doxorubicin. Degradation of organic nanocarriers into components with negligible toxicity which is cleared through circulation easily is an added advantage [19]. Some of these nanoparticles like liposomes contain aqueous and hydrophobic phases allowing accommodation of hydrophilic and lipophilic substances simultaneously [20]. Organic nanoparticles may be synthesized using polymorphic lipids which enable stimulus responsive release; however, an inorganic component may be added to supplement the therapeutic effects of encapsulated cargo. For example, Shen *et al*., synthesized superparamagnetic iron oxide (Fe_3O_4) nanoparticles loaded with thermosensitive liposomes to impart synergistic photothermal therapy and chemotherapy [21]. Similarly magnetic PLGA nanoparticles were synthesized for chemotherapy and magnetic resonance imaging whereas, unimolecular micelles conjugated to gold (Au) nanoparticles were used for computed tomography imaging in addition to drug delivery [22,23].

Inorganic nanoparticles have been used for various applications in addition to drug delivery such as thermal ablation be it in the form of photothermal therapy (PTT) or magnetic hyperthermia, photodynamic therapy (PDT), sonodynamic therapy (SDT), optical imaging, magnetic resonance imaging (MRI), computed tomography (CT), photoacoustic tomography (PAT) so on and so forth. For example, Caro *et al*., synthesized Fe_3O_4-Au nanoparticles for

PTT guided by dual mode MRI and CT imaging [24]. NIR and ultrasound-responsive $MnFe_2O_4$/C nanocomposites for photodynamic and sonodynamic therapies in addition to magnetic resonance imaging whereas, gadolinium (Gd) doping to Fe_3O_4 nanoparticles was performed to enhance its MRI contrast [25,26]. Thus, while organic nanoparticles are highly biocompatible and have been used for delivery, inorganic nanoparticles can themselves be used as mediators of therapy and imaging without the addition of other molecules.

2.3. Favorable properties of inorganic nanoparticles:

Intrinsic properties of inorganic nanoparticles which arise at the nanoscale and their applications are described in this section. Iron oxide nanoparticles exhibit high magnetization and negative MRI contrast resulting in applications for magnetic hyperthermia, magnetically controlled drug delivery and MRI [27]. Au nanoparticles are used in treatment modalities such as PTT, PDT, sonodynamic therapy and imaging methods like CT, PAT owing to their ability to absorb NIR irradiation and generate different responses, surface plasmon resonance and high X-ray absorption co-efficient [28]. PTT, PAT applications of copper sulfide (CuS) nanoparticles stem from its ability to absorb NIR and generate heat. This effect in CuS nanoparticles arises from d-d transitions of electrons [29,30]. Quantum confinement effects of quantum dots and zinc-based nanoparticles have enabled their use in optical imaging techniques [31]. Doping with metals like Gd and manganese (Mn) can confer properties like superparamagnetism which can translate into MRI and magnetic hyperthermia applications [32]. NanoTherm, Hensify and AuroShell are some inorganic nanoparticles exploiting above mentioned physical properties and awaiting FDA approval for magnetic hyperthermia, radiosensitization and PTT of cancers [33].

Chapter 3: Rationale, Aim and Objectives

3.1. Rationale:

Cancer continues to be a major health burden globally. Intratumoral heterogeneity and resistance to chemotherapeutic drugs limits disease cure and affects patient outcome. Despite innovations in the development of new treatment modalities, these treatments still show low effectiveness and neither have they led to substantial changes in the overall 5-year survival rate of cancer patients. For example, immune checkpoint blockers which are the most commonly used form of cancer immunotherapy show response rates of below 30% in patients overall [34]. Thus, there is a growing need for alternative treatment strategies which can overcome

limitations of traditional methods and at the same time overcome resistance often observed in cancers. The nanomedicine landscape is a continuously evolving one showing great potential to reform cancer treatment. A majority of nanomedicines approved for cancer treatment include pre-approved chemotherapeutic drugs encapsulated in respective nanoparticles, which rarely exploit the novel chemical, magnetic, morphological characteristics observed at the nanoscale [35]. We aimed to develop a nanoparticle system composed of inorganic materials capable of imparting treatment to cancer cells by utilizing the intrinsic properties of the material described earlier. Thus, we chose a CuS-ZnS nanocomposite system as CuS shows photothermal behavior whereas, ZnS has been declared Generally Regarded As Safe (GRAS) by FDA and has been utilized for imaging. We also used this system to deliver chemotherapeutic drugs which in addition to the PTT would maximize therapeutic efficiency in terms of killing cancer cells.

3.2. Aim:

To synthesize and design surface functionalized hybrid metal/metal oxide nanoparticles for therapeutic and bio-imaging applications

3.3. Objectives:

- Synthesis and characterization of hybrid metal/metal sulfide nanoparticles
- Surface functionalization and attachment of targeting moieties
- Dual drug encapsulation
- Assessment of stimulus responsive performance of nanoparticles
- Assessment of biocompatibility, photothermal behavior of nanoparticles *in vitro*
- Assessment of acute toxicity of nanoparticles by *in vivo* studies

Chapter 4: Synthesis and characterization of folate functionalized, dual drug encapsulated chitosan coated CuS-ZnS (FDDCh-CZ) nanocomposites

Copper sulfide-zinc sulfide nanocomposites (CuS-ZnS NC) were synthesized by a hydrothermal route under constant stirring. Surface modifications like coating with biocompatible polymer (chitosan) and attachment of targeting moiety (folate) was performed *in situ*. Synthesized nanocomposites were characterized for successful nanocomposite formation [U.V-Visible spectroscopy, X-Ray photoelectron spectroscopy (XPS) and Energy dispersive X-Ray spectroscopy (EDX)], functional groups [Fourier transform Infrared spectroscopy (FT-IR)], surface charge [Zeta potential], photoluminescence

[Photoluminescence spectroscopy], size distribution and morphology [High Resolution Transmission Electron Microscopy (HRTEM)] and crystalline nature [X-Ray diffraction (XRD)], respectively.

4.1. Synthesis of CuS-ZnS nanocomposite (CZ NC):

CZ NC was synthesized using copper (Cu), zinc (Zn) and sulphur (S) precursors *via* hydrothermal route using the method described by Feng *et al.,* with some modifications [36]. Briefly, Cu, Zn and S precursors in a stoichiometric ratio of 1:1:2 were added to a beaker containing 0.02 mmol/L sodium chloride (NaOH) solution placed on a magnetic stirrer and were allowed to react for 2 hours at 60 °C. Thereafter, a black colored solution containing CZ NC was obtained and the nanoparticles were procured by centrifugation. This was followed by washing the NC with distilled water (D/W) repeatedly and drying in the oven for 24 hours. Individual CuS and ZnS nanoparticles were prepared by the same method using their respective precursors respectively.

4.2. Synthesis of chitosan coated CZ NC (Ch-CZ NC):

Ch-CZ NC were obtained using the hydrothermal method as described above. In brief, Cu, Zn and S precursors in a stoichiometric ratio of 1:1:2 were added to a beaker containing 0.02 mmol/L sodium chloride (NaOH) solution placed on a magnetic stirrer and were allowed to react for 2 hours at 60 °C. To the black colored solution, chitosan prepared in 2% (v/v) acetic acid was added dropwise and allowed to stir for 2 hours at 60 °C. Obtained Ch-CZ NC were rinsed multiple times using D/W by centrifuging for 10 min at 10,000 rpm. Obtained nanoparticles were dried at room temperature.

4.3. Encapsulation of dual drug in Ch-CZ NC (DDCh-CZ NC):

Chitosan addition was followed by encapsulation of model hydrophilic [5-Fluorouracl (5 FU)] and hydrophobic [Paclitaxel (Ptxl)] drugs. Addition of 5 FU was followed by the addition of Ptxl. Each step was followed by a step of washing to remove unbound excess drugs by centrifugation at 10,000 rpm for 10 min.

4.4. Attachment of folate moieties on DDCh-CZ NC (FDDCh-CZ NC):

Dual drug encapsulated NC from the above step were then re-suspended in 0.02 mmol/L NaOH solution followed by the addition of folate prepared in NaOH solution (pH 10). The reaction was carried out overnight, under dark conditions. Obtained FDDCh-CZ NC were separated

from the reaction solution by centrifugation followed by D/W rinse at 10,000 rpm for 10 min and finally dried overnight at room temperature.

4.5. Characterization of FDDCh-CZ NC:

4.5.1. U.V-Visible spectroscopy:

U.V-Visible spectroscopy confirmed the formation of the nanocomposite which was observed as peaks at 214 nm representing ZnS and 808 nm representing CuS. Compared to individual CuS and ZnS nanoparticles, peaks showed a hypsochromic shift which could be indicative of re-arrangements of molecules occurring during NC formation on the surface [37,38]. Peaks for folate were observed at 279 nm and 364 nm indicating successful attachment of folate on the surface of the NC [39].

4.5.2. FT-IR:

FT-IR spectroscopy was used to confirm chitosan coating over the nanocomposite observed by the presence of its functional groups in the spectrum. Peaks for N-H and O-H stretching were observed at 3783 cm^{-1} and 3429 cm^{-1}. C-H symmetric and asymmetric stretching was observed at 2916 cm^{-1} and 2850 cm^{-1} respectively. Peaks for C=O stretching, CH_2 bending and C-O stretching were observed at 1628 cm^{-1}, 1401 cm^{-1} and 1051 cm^{-1} respectively [40]. Additionally, peaks for the core were also observed at 1109 cm^{-1} and 616 cm^{-1} which correspond to ZnS and Cu-S stretching respectively [41,42].

4.5.3. XPS:

To examine chemical composition and the elemental status of Cu, Zn and S in the prepared NC, XPS spectroscopy was carried out. In XPS spectrum of CuS nanoparticles, binding energy peaks for Cu $2p_{3/2}$ and $2p_{1/2}$ were observed at 942 eV and 962 eV with a difference of 20 eV between them which is characteristic of Cu in CuS. Binding energy for S was observed at 164 eV. Binding energy peaks observed at 1022 eV and 1045 eV were identified as Zn $2p_{1/2}$ and $2p_{3/2}$ in XPS spectrum of ZnS nanoparticles. Whereas, peaks for S were observed at 157 eV and 161 eV. In XPS spectra of CZ NC, binding energy peaks for Cu, Zn and S were observed. Slight shift in the energies for Zn was observed which may result from the exchange of electrons on their surface resulting from the differences in electronegativity between Cu and Zn [43].

4.5.4. Zeta Potential:

Zeta potential analysis of the different nanoparticle systems was assessed to determine their surface charge. The results showed that successful coating of chitosan over the NC could result from electrostatic interactions between negatively charged NC and positively charged chitosan at neutral pH.

4.5.5. XRD:

XRD analysis of the different nanoparticle system was performed to determine their crystalline nature. 2θ values of 28.22°, 48.13° and 57.50° were indexed to (111), (220) and (311) planes of ZnS (Zn blende) whereas, 22.42, 29.36, 31.80, 48.21, 52.83 and 59.43 were indexed to (100), (102), (103), (110), (114) and (203) planes of covellite phase of CuS [44,45].

4.5.6. HRTEM:

HRTEM analysis was perform to determine the size distribution and morphological characteristics of the nanoparticles. The NC showed a mixed morphology of spherical and rod-shaped particles having a size distribution of 2-12 nm. However, after coating this range shifted to 7-16 nm and the presence of a layer of coating over the NC was clearly observed. d-spacing values of 0.028 nm and 0.3 nm were observed which correspond to CuS and ZnS respectively. Selective area electron diffraction (SAED) pattern showed the presence of planes indexed in XRD.

4.5.7. EDX:

EDX analysis was performed analyze the elemental composition of the NC which showed the presence of Cu, Zn and S peaks.

4.6. Discussion:

This study reports the facile synthesis of an inorganic nanocomposite system for encapsulation of dual drug and photothermal therapy. The probable mechanism for NC formation is the differences in solubility product of CuS and ZnS. As the solubility product of ZnS is higher than that of CuS, Zn^{2+} and S^{2-} ions from the formed ZnS nanoparticles constantly dissolve into the surrounding medium which are then taken up by Cu^{2+} ions to form CuS [46]. An alternate mechanism is the cation exchange theory which suggests ion exchange as the driving force. According to this theory, S^{2-} ions present around ZnS are highly likely to react with Cu^{2+} ions in the solution. As a result, a thin shell of CuS nanocrystals is deposited by heterogenous nucleation on the surface of ZnS [47]. The presence of ZnS results in photoluminescence as

was observed from the photoluminescence spectroscopy results. Further chapters also show the photothermal behavior of the nanocomposite which results from the photothermal nature of CuS. Chitosan was selected as the coating material due to its biocompatible nature and amine groups having pKa ~6.5. As a result of such favorable properties, chitosan has been used in several studies for delivery of hydrophobic and hydrophilic drugs [48]. Folate was selected as the targeting ligand as several studies have reported over-expression of the folate receptor in various cancers [49]. Thus, by attachment of a folate moiety on the surface of a nanoparticle, one can target cells towards folate receptor positive tumor cells which would also allow folate receptor mediated endocytosis of the nanoparticles [50]. Thus, materials selected for the modification of the inorganic core are such that they would add to the functionality and biocompatibility of the nanocomposite.

Chapter 5: Drug encapsulation and release studies

5.1. Dual drug encapsulation:

Chemotherapy which is the use of drugs to kill cancer cells is a commonly used treatment modality for cancer. However, when treated with a drug (also known as monotherapy), cancer is known to develop resistance to the drug over a period of time by different mechanisms such as increasing efflux or decreasing influx of the drug by alteration of transporter levels, modification of drug specific targets to make drug ineffective, increased metabolism of drug etc. Also, as a result of the tumor microenvironment heterogeneity and complexity, a variety of such resistance mechanisms may be exhibited by tumor cells [51]. In order to overcome resistance one of the strategies used is treatment with a combination of two or more therapeutic agents (also known as combination therapy or dual drug therapy) [52]. Combination therapy has resulted in improved treatment, dose reduction and also reduction in drug resistance as multiple pathways are simultaneously targeted [53].

In this study, we have encapsulated two drugs: hydrophilic 5 FU and hydrophobic Ptxl. 5 FU is a pyrimidine analog, acts by interfering with DNA synthesis and has λ_{max} of 266 nm [54]. Ptxl is a plant alkaloid and an anti-microtubule agent with λ_{max} of 228 nm [55].

5.1.1 Drug encapsulation studies:

As mentioned earlier, anti-cancer drugs 5 FU and Ptxl in a 1:1 ratio was encapsulated *in situ*. 2 hours after the addition of chitosan was followed by the addition of 5 FU which was allowed

to react for 20 min under constant stirring at room temperature. The nanocomposites were then washed using D/W to remove the excess unbound drug. To re-suspended nanocomposites, Ptxl was added and encapsulation was performed under constant stirring for 1 hour at 40 °C. This step was followed by a D/W rinse. Supernatant from both the washing steps was used to calculate the encapsulation efficiency (% EE) by U.V-Visible spectroscopy and the following formula was used [56]:

$$\% \text{ EE} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

% EE was calculated by using the standard curve analysis and was found to be 97.63% for 5 FU and 41.88% for Ptxl respectively.

5.2. Dual drug release:

Drug release from the nanocomposites was analyzed using the dialysis bag method which is based on the diffusion of solutes across a semi-permeable membrane from a concentrated to a lower concentrated solution, until equilibrium is attained. Aliquots were taken every hour upto 8 hours followed by sampling at 24, 48 and 72 hours. Release was studied at pH 5 and 7 in phosphate buffered saline (PBS) and simulated body fluid (SBF) having a pH of 7.4 [57]. Drug release of the free drugs and dual drug encapsulated NC was studied.

5.2.1. Dual drug release studies:

Free drug showed ~100% release in hours, whereas 5 FU and Ptxl showed highest release in acidic pH. 69% of 5 FU and 28% Ptxl was released in acidic pH at 72 hours whereas, in pH 7 40% and 4.6% release for 5 FU and Ptxl was observed respectively. Release in SBF showed release pattern similar to that of PBS pH 7. Highest release observed in acidic pH indicated the NC system was capable of a stimulus-responsive release.

5.3. Mathematical fitting of release data:

Mathematical fitting of obtained release data was performed to examine the mechanism of drug release from the nanocomposites. Different kinetic models such as 0, 1st and 2nd order models and also Korsmeyer-Peppas, Higuchi and Hixson-Crowell models were tested. Highest co-relation co-efficients for 5 FU and Ptxl were obtained in 0 order model at pH 5 and pH 7 regardless of the release medium.

5.4. Discussion:

Dual drug encapsulation and stimulus-responsiveness were both enabled by the use of chitosan. Negatively charged 5 FU is known to interact electrostatically with positively charged chitosan whereas, Ptxl is known to interact with chitosan by π-π stacking [58,59]. Stimulus-responsive release of these drugs arises from the presence of pH ionizable amine groups of chitosan. Under acidic pH these amine groups are protonated causing charge repulsion and thus opening up of the polymer chain thus enabling drug release. Under alkaline or neutral pH conditions, these groups are deprotonated causing the polymer to shrink and thus restricting movement of drug molecules [60]. Thus, the nanocomposite enabled drug release in acidic pH which would enable tumor microenvironment specific release which is also known to be acidic.

Chapter 6: Assessment of biocompatibility of the nanocomposites

6.1. Nanomaterial-cellular interactions and their uptake:

Increasing use of nanoparticles for biomedical applications has led to growing concern over their potential cytotoxic effects. Once injected *in vivo*, nanoparticles may interact physically and chemically with cells, biological molecules which could also affect their biological fate and therapeutic effects [61, 62]. In order to evaluate biocompatibility and cytotoxic effects of fabricated nanocomposites, we assessed their effects on normal and cancer cells by MTT assay in a concentration and time dependent manner. This was followed by assessment of haemocompatibility as a measure of haemolytic effect of nanocomposites on red blood cells. As *in vitro* studies cannot always simulate *in vivo* conditions, we concluded biocompatibility assessments with acute toxicity study which was conducted using female Wistar rats following OECD guidelines.

6.1.1. Maintenance of cells:

Mouse embryonic fibroblast cell line (NIH-3T3), human cervical cancer cell line (HeLa), mouse breast cancer cell line (4T1) and human breast cancer cell line (MCF-7) were used for cell culture studies. NIH-3T3 and HeLa were used for assessment of cytotoxicity whereas, MCF-7 was used to study *in vitro* PTT effect. All cell lines were maintained at 37 °C and 5% CO_2 and cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% PenStrep.

6.1.2. Assessment of *in vitro* cytotoxicity by MTT assay:

Cellular toxicity induced by FDDCh-CZ, Ch-CZ and CZ NC was assessed quantitatively in a dose dependent manner for 24, 48 and 72 hours using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay. Cells were treated with 50, 100, 200, 250 and 300 μg/mL NC, which was followed by the addition of the MTT reagent. After incubation for 4 hours at 37 °C, 200 μL of dimethyl sulfoxide (DMSO) was added and absorbance was recorded at 570 nm and 655 nm using Bio-Rad Model 680 microplate reader. % Viability was calculated using the formula:

$$\% \text{ Viability} = \frac{\text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

The NC showed a time and dose dependent cytotoxic effect on the cells. Ch-CZ NC showed higher compatibility compared to CZ and FDDCh-CZ NC which may result from the chitosan coating over the NC. 300 μg/mL FDDCh-CZ NC led to inhibition of >90% Hela cells whereas, ~40% viability of NIH-3T3 was observed after 72 hours of treatment. These results indicated that FDDCh-CZ NC showed higher toxicity in HeLa as compared to NIH-3T3 cells.

6.2. Haemocompatibility studies:

Nanoparticles meant for therapeutic applications would at some point come with contact with blood and thus it is necessary to evaluate their effect on blood. Haemocompatibility studies are conducted by contacting materials to be tested with blood and/or components of blood [63]. As red blood cells (RBCs) are the most abundant cell type found in blood, we have evaluated the haemocompatibility of the NC as a measure of their hemolysis effect. All experiments were conducted after approval from Institutional Ethics Committee (IEC Approval number: NMIMS/IEC/013/2020). To test the haemocompatibility, RBCs from ethylenediamine tetraacetic acid (EDTA) stabilized blood collected from healthy donors (n=6) were collected by the Ficoll-Hypaque method and were subsequently washed using PBS (pH 7). PBS was also used to dilute the obtained RBC pellet and make FDDCh-CZ NC solutions in the 50-300 μg/mL concentration range. 0.8 mL NC were then incubated with 0.2 mL RBC suspension for 2 hours at 37 °C with intermittent shaking. RBCs incubated with Triton X and PBS were maintained as positive and negative controls respectively. After incubation, the samples were centrifuged at 1200 rpm for 5min and obtained supernatant was then analyzed at 570 and 655 nm using an ELISA reader [64].

FDDCh-CZ NC tested in the 50-300 μg/mL did not show any visible haemolysis as compared to the positive control. Also, the % haemolysis calculated was well below 5 % indicating their haemocompatible nature.

6.3. Cellular uptake studies

Cellular uptake studies were performed using NIH-3T3 and HeLa cell lines. Cells were seeded in 60 mm petri dishes at a density of 1×10^6 cells/dish and were allowed to grow overnight in an incubator set at 37 °C in the presence of 5% CO_2. The following day growth medium was replaced with serum free medium containing FDDCh-CZ NC (200 μg/mL) and uptake was allowed to take place over 24, 48 and 72 h respectively. Incubation for respective time periods was followed by PBS wash and cells were collected by trypsinization. Obtained cell pellet was digested using 3 mL aqua regia and analyzed by ICP-AES technique to quantitatively measure cellular uptake of FDDCh-CZ NC.

Higher uptake of FDDCh-CZ NC was observed in HeLa cells as compared to NIH-3T3 which corroborated results observed in MTT assay.

6.4. *In vivo* acute toxicity studies

Acute toxicity studies were conducted *in vivo* as per OECD 425 guideline for acute oral toxicity up-and-down procedure after approval from Institutional Animal Ethics Committee (Approval number: CPCSEA/IAEC/P-14/2020). The toxicity of 55, 175 and 575 mg/kg of FDDCh-CZ NC was tested in female Wistar rats which were randomized into three groups. For the initial three hours, changes in behavior of the animals were assessed. This was followed by monitoring of changes in their body weight, eye movement, palpitation, eye color, vomiting, diarrhea, writhing effect, convulsions, tremors, excreta, food and water intake, salivation, color of fur and any other symptoms of toxicity or mortality every day for a period of 14 days.

No changes in the body weight, eye movement, palpitation, eye color, vomiting, diarrhea, writhing effect, convulsions, tremors, excreta, food and water intake, salivation, color of fur was observed, neither did the animals show any signs of toxicity or mortality.

6.5. Discussion:

The *in vitro* compatibility of synthesized FDDCh-CZ NC was evaluated by haemocompatibility and cytotoxicity studies whereas *in vivo* assessment was performed by acute toxicity study. The NC showed no haemolytic effect on RBCs which indicated good haemocompatibility, whereas higher cytotoxicity was noticed in HeLa cells as compared to

NIH-3T3 cells. The specific higher toxicity in HeLa cells may arise from the targeted uptake of the NC by folate receptor mediated endocytosis which was confirmed by cellular uptake studies. Cumulative results from the *in vitro* and *in vivo* studies clearly indicate the haemocompatible and biocompatible nature of the NC. Toxicity concerns with respect to inorganic nanoparticles is a major cause of concern for their use in clinical settings. However, from our studies, the synthesized NC did not show significant signs of toxicity or incompatibility *in vitro* and *in vivo*.

Chapter 7: Assessment of theranostic applications of nanocomposite

7.1. Theranostic properties:

Theranostics refers to the use of a material for therapeutic and diagnostic applications simultaneously. Nanoparticles as a result of their favorable properties and ability to accumulate in small spaces are increasingly being developed as theranostic systems. In this study, we developed a theranostic system using two metal sulfides i.e., CuS and ZnS. CuS was chosen for its excellent photothermal properties whereas ZnS was chosen for its photoluminescence.

7.1.1. Photothermal therapy

Photothermal therapy makes use of photothermal agents which absorb energy from a source (usually NIR lasers) and convert this energy into heat (also known as PTT behavior). This process is called hyperthermia under clinical settings. It is a local and minimally invasive treatment modality. Hyperthermia can lead to many harmful effects such as lysis of cell membrane, denaturation of proteins and evaporation of cytoplasm which can ultimately result in death [65]. Inorganic nanoparticles based on CuS, Au are known to possess the ability to absorb NIR light and generate heat. This heat is also known to make the cancer cells sensitive toward other treatments. The PTT behavior in CuS nanoparticles arises from intraband d-d transitions which unlike surface plasmon resonance of Au nanoparticles is independent of its surrounding medium [66].

7.1.2. Photoluminescence

Photoluminescence (PL) refers to the process of absorption of light followed by emission from the excited state. Semiconductor materials like ZnS exhibit tunable photoluminescence which finds applications in bio-imaging, UV photodetectors, flat panel displays etc. PL in ZnS arises from non-radiative decay of excited electrons and can be tuned by variable doping of elements.

As a result of such favorable properties and "Generally Regarded as Safe" status by FDA, we incorporated ZnS in the nanocomposite [67].

7.2. Characterization of photoluminescent behavior

Photoluminescent behavior of ZnS was qualitatively and quantitatively analyzed as stated below

7.2.1. Photoluminescence spectroscopy

Photoluminescence spectra of ZnS NPs and CZ NC were recorded using Princeton Instruments Acton SP2300 photoluminescence spectrofluorophotometer by exciting the nanoparticles at 350 nm.

Photoluminescence spectra showed peak for ZnS nanoparticles at 464 nm which was red shifted in the NC. This shift could arise from inhibition of active luminescent centers of ZnS due to the presence of CuS [68].

7.2.2. Estimation of photoluminescence

PL of FDDCh-CZ NC was quantitatively evaluated using BioTek Cytation 5 Imaging Reader with excitation and emission at 350 and 560 nm respectively. PL measurements of FDDCh-CZ NC suspended in incomplete Roswell Park Memorial Institute medium (I-RPMI) and relative fluorescence was quantified in comparison to negative control.

The PL of FDDCh-CZ NC increased in a concentration dependent manner with 20 μg showing PL signal of 10,000 RFU whereas 200 μg showed PL signal of 15,000 RFU.

7.3. Photothermal transduction studies

Photothermal conversion efficiency (PCE) is the ability of a material to convert light into heat. Prior to calculating the PCE of the NC, we performed a pre-liminary testing to determine the concentration of NC which would give a suitable increase in temperature. For this, different concentrations of the NC were exposed to a continuous wave 808 nm laser and the rise in temperature was recorded using a probe thermometer 4 min after irradiation. The concentration of 200 μg/mL of the NC showed temperature rise in the 42-48 °C temperature range which is recommended for PTT. Thus, all further PTT experiments were conducted using a concentration of 200 μg/mL of the NC.

The PCE was assessed by exposing the NC solution to 808 nm laser irradiation until temperature saturation i.e., a constant temperature was attained. Temperature of the solution

was monitored before the start of the experiment and thereafter every 15 sec. D/W was used as control [69].

Maximum temperature (T_{max}) of the NC i.e., 53.8 °C was attained in 18 min as compared to T_{max} of D/W i.e., 35.4 °C which was attained in 16.75 min. PCE of the NC was calculated as 40.15%.

7.4. *In vitro* PTT efficacy studies

To assess the PTT effect of the NC *in vitro*, 200 μg/mL FDDCh-CZ NC was incubated with MCF-7 cells followed by 808 nm continuous-wave laser treatment for 5 min followed by incubation for 2 hours. Viability of the cells was then evaluated by Alamar blue assay. Controls maintained for the experiment included cell control (only cells), material control (cells + FDDCh-CZ) and laser control (cells + laser). % Viability was calculated using the formula mentioned above, by recording absorbance at 600 nm with 570 nm as reference filter.

While all the controls showed viability above 90%, the cells treated with FDDCh-CZ NC and exposed to laser for 5 min showed viability below 20% indicating effective PTT therapy induced by the NC.

7.5. *In vivo* therapeutic efficacy of FDDCh-CZ NC and bioluminescence imaging:

Therapeutic efficacy of synthesized FDDCh-CZ was evaluated in clinical tumor model using CD1 mice. Tumor was induced by injecting 4T1Luc2 cells expressing firefly luciferase enzyme and therapy was administered once the tumor attained a volume of 70 ± 10 mm^3 (average radiance value ~ $2\text{-}3\times10^9$). Study groups consisted of mice randomly distributed into 3 groups *viz.* untreated control, material control and PTT treatment group. Untreated control received incomplete RPMI medium and material control was administered FDDCh-CZ NC without laser treatment. The PTT treatment group on the other hand received FDDCh-CZ NC and was exposed to 808 nm laser treatment for 5 min for 2 days consecutively. Therapeutic efficacy was monitored in terms of tumor inhibition or growth by bioluminescence imaging (BLI) using D-luciferin potassium salt. BLI images were captured using PerkinElmer IVIS Lumina *in vivo* imaging system and average radiance was obtained using Living Image v4.4 software.

PTT treatment group showed a reduction in BLI signal from 3×10^9 (control) to 2.5×10^9 on day 1 post PTT treatment. After the second booster PTT treatment, significant reduction in tumor BLI signal was observed. On the other hand, untreated control and material control groups showed no decrease in BLI signal. BLI signal increased from 1.0×10^9 on day 0 to 4.0

$\times 10^9$ on day 5 for untreated control group whereas material control showed increase from 1.5×10^9 on day 0 to 6.1×10^9 on day 5.

7.5. Discussion:

Development of photo-controlled therapies has gained increasing interest in recent years as they offer the advantage of targeted spatial and temporal accuracy upon irradiation with light of specific wavelength. In this study, we have developed a nanomaterial system which photoluminescent and responsive to NIR in the first biological window i.e., 750-950 nm also known as NIR-I. Pl behavior was exhibited by the nanocomposites after excitation at 350 nm however the peak showed a red shift in comparison to ZnS NPs. Quantitative estimations showed potential for the nanocomposite to be used as an imaging or diagnostic agent. On the other hand, upon exposure to laser at 808 nm the NC is capable of heat generation which was further exploited as a means to deliver PTT to cells. Also, the NC showed a PCE of ~41% which is sufficient for efficient PTT of cancer cells. CuS was chosen as the material to deliver PTT as it is economical, easy to synthesize and shows PTT behavior independent of its surrounding medium. When cells were incubated with 200 μg/mL FDDCh-CZ NC for 2 hours and laser it led to a >80% viability in cells. On the other hand, cells treated with FDDCh-CZ NC for 24 hours did not show significant decrease in viability. This significant decrease in viability after laser treatment can result from synergistic chemo-PTT effect. At 5 min after laser irradiation, the temperature of the NC increases to 46.8 °C as was determined from PCE studies. Irreparable damage to cells ensues which also increases their susceptibility to other forms of treatment [70]. Further, we evaluated the therapeutic efficacy of FDDCh-CZ NC *in vivo* in immunodeficient mice. Significant reduction in tumor mass was observed post PTT treatment as compared to the untreated and material control groups. Thus, the increased inhibition could be a collective effect of PTT and chemotherapy. These results also highlight the excellent PTT properties of the NC and their potential to be used as PTT agents.

Chapter 8: Summary

Inorganic nanocomposite system composed of CuS-ZnS were successfully synthesized using a facile hydrothermal route. The synthesized NC were folate functionalized, chitosan coated and dual drug encapsulated *in situ*. Various characterization techniques like U.V-Visible spectroscopy, FT-IR, XRD, XPS and EDX analysis showed successful formation of the nanocomposite. U.V-Visible spectroscopy showed successful folate functionalization over the

NC and peaks for CuS, Zns. Presence of functional groups of chitosan was observed in FT-IR spectrum of the NC which confirmed its coating over the NC. Zeta potential analysis showed probability of electrostatic interactions between chitosan and the NC which resulted in successful coating. XRD analysis indicated the presence of covellite and Zn blende phases of Cu and Zn respectively and maintenance of the crystalline nature of the NC after chitosan coating. HRTEM analysis showed the presence of mixed morphology of the NC with size distribution ranging from 7-16 nm of the FDDCh-CZ NC. Dual drug encapsulation and stimulus responsive nature the NC was enabled by chitosan coating on the NC. Encapsulation efficiencies of 98% and 42% were attained for 5 FU and Ptxl respectively. Drug release studies showed higher drug release of 5 FU and Ptxl in acidic medium as compared to neutral pH and mathematical model fitting indicated that drug release from the NC followed zero order kinetics. MTT assay showed a time and dose dependent cytotoxic effect of the NC over cells and higher toxicity was observed in HeLa cells as compared to NIH-3T3. Hemocompatibility results showed FDDCh-CZ NC up to concentrations of 300 μg/mL showed hemolysis below 5% which a good indicator of their hemocompatibility. Cellular uptake studies showed higher uptake of FDDCh-CZ NC by cancer cells as compared to normal cells. No signs of toxicity or mortality were observed for 55, 175 and 575 mg/kg tested doses of FDDCh-CZ NC in acute toxicity *in vivo* studies. Photoluminescence spectroscopy demonstrated the potential of the NC for photoluminescence whereas PTT experiments showed PCE of the NC was 41%. Excellent photothermal properties translated into 80% cell death for 200 μg/mL of FDDCh-CZ NC *in vitro*. *In vivo*, significant destruction of tumor was observed in terms of reduction in bioluminescence intensity post PTT treatment indicative of good therapeutic efficacy of the fabricated FDDCh-CZ NC system.

Conclusions:

Thus, we conclude that the synthesized FDDCh-CZ NC shows potential for use as a PTT agent, deliver chemotherapeutic drugs and prospects for photoluminescence. The NC showed highest release of drugs in acidic pH indicative of their stimulus-responsive nature which can translate into selective release in the acidic environment of the tumor. The NC also showed targeted delivery as was indicative of higher toxicity observed in cancer cell line. Haemocompatibility and acute *in vivo* toxicity studies showed good compatible nature of the NC.

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